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# Junctional Adhesion Molecule A Serves as a Receptor for Prototype and Field-Isolate Strains of Mammalian Reovirus

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**Reovirus infections are initiated by the binding of viral attachment protein σ1 to receptors on the surface of host cells. The σ1 protein is an elongated fiber comprised of an N-terminal tail that inserts into the virion and a C-terminal head that extends from the virion surface. The prototype reovirus strains type 1 Lang/53 (T1L/53) and type 3 Dearing/55 (T3D/55) use junctional adhesion molecule A (JAM-A) as a receptor. The C-terminal half of the T3D/55 σ1 protein interacts directly with JAM-A, but the determinants of receptor-binding specificity have not been identified. In this study, we investigated whether JAM-A also mediates the attachment of the prototype reovirus strain type 2 Jones/55 (T2J/55) and a panel of field-isolate strains representing each of the three serotypes. Antibodies specific for JAM-A were capable of inhibiting infections of HeLa cells by T1L/53, T2J/55, and T3D/55, demonstrating that strains of all three serotypes use JAM-A as a receptor. To corroborate these findings, we introduced JAM-A or the structurally related JAM family members JAM-B and JAM-C into Chinese hamster ovary cells, which are poorly permissive for reovirus infection. Both prototype and field-isolate reovirus strains were capable of infecting cells transfected with JAM-A but not those transfected with JAM-B or JAM-C. A sequence analysis of the σ1-encoding S1 gene segment of the strains chosen for study revealed little conservation in the deduced σ1 amino acid sequences among the three serotypes. This contrasts markedly with the observed sequence variability within each serotype, which is confined to a small number of amino acids. Mapping of these residues onto the crystal structure of σ1 identified regions of conservation and variability, suggesting a likely mode of JAM-A binding via a conserved surface at the base of the σ1 head domain.**

Mammalian orthoreoviruses (referred to as reoviruses in this article) are nonenveloped viruses with genomes of 10 discrete segments of double-stranded RNA (reviewed in reference 41). There are at least three serotypes of reoviruses, which can be differentiated by the capacity of antireovirus antisera to neutralize viral infectivity and inhibit hemagglutination (47, 50). Each of the reovirus serotypes is represented by a prototype strain, namely, type 1 Lang/53 (T1L/53), type 2 Jones/55 (T2J/55), and type 3 Dearing/55 (T3D/55). Reoviruses appear to infect most mammalian species, but disease is restricted to the very young (reviewed in reference 63). Reovirus infections of newborn mice have been used as the preferred experimental system for studies of reovirus pathogenesis. Sequence polymorphisms in reovirus attachment protein σ1 play an important role in determining sites of reovirus infection in the infected host (4, 32, 69, 70).

The σ1 protein is an elongated trimer with a head-and-tail morphology. The N-terminal σ1 tail partially inserts into the virion via "turrets" formed by the pentameric λ2 protein, while the C-terminal σ1 head projects away from the virion surface (1, 25, 26). A crystal structure of the C-terminal half of T3D/55 σ1 revealed that the head contains three β-barrel domains (one from each trimer), each of which is constructed from eight antiparallel β-strands (16). Sequence analysis and structural modeling have suggested that the N-terminal half of the tail is formed from an α-helical coiled coil (6, 21, 40) and the C-terminal half is formed from a triple β-spiral (16, 56). The overall structural topology of the β-spiral and head domains of σ1 is strikingly similar to that of the adenovirus attachment protein, fiber (16, 37, 56).

There are two distinct receptor-binding regions in σ1. A region in the fibrous tail domain of type 3 σ1 binds to α-linked sialic acid (2, 14, 15, 18). A distinct region in the type 1 σ1 tail domain also binds to cell surface carbohydrates (14), and recent evidence suggests that sialic acid may be involved in the binding of T1L/53 to intestinal cells (30). A second receptor-binding site is located in the head domains of both the type 1 and type 3 σ1 proteins (3, 39).

An expression-cloning approach was used to identify junctional adhesion molecule A (JAM-A) as a receptor for the prototype strains T1L/53 and T3D/55 (3). JAM-A is a 35-kDa type I transmembrane protein that is a member of the immunoglobulin superfamily (34, 36). JAM-A contains two immunoglobulin-like domains, a single transmembrane region, and a short cytoplasmic tail. JAM-A is expressed in a variety of

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tissues, including epithelial and endothelial barriers (34, 36, 43), where it is thought to regulate tight-junction permeability and mediate leukocyte trafficking (17, 34, 36, 43).

The crystal structures of the murine (m) and human (h) homologs of JAM-A, both of which are functional reovirus receptors (3), indicate that JAM-A forms homodimers via extensive hydrophobic and ionic contacts between apposing membrane-distal (D1) immunoglobulin-like domains (33, 44). Residues that facilitate interdimer interactions are strictly conserved between mJAM-A and hJAM-A (33, 44). JAM-A dimers are thought to be physiologically relevant, perhaps functioning in tight-junction barrier integrity or the diapedesis of inflammatory cells (8, 33, 44). Recent biochemical studies of reovirus–JAM-A interactions suggested that σ1 binds to a monomeric version of JAM-A and contacts residues in the vicinity of the JAM-A dimer interface (24). This strategy of cell attachment is strikingly similar to that used by adenovirus fiber to bind to the coxsackievirus and adenovirus receptor (CAR) (10, 66), an immunoglobulin superfamily member that shares considerable structural homology with JAM-A (57).

For this study, we determined whether JAM-A serves as a receptor for the prototype type 2 strain T2J/55 and a panel of four type 1, two type 2, and four type 3 field-isolate strains. The results indicate that JAM-A, but not the related JAM family members JAM-B and JAM-C, is a receptor for prototype and field-isolate strains of the three reovirus serotypes. An analysis of conserved and variable sequences in the σ1 head, together with existing structural information for σ1 and JAM-A, suggested an especially high tolerance for surface variation in the protein while maintaining the specificity for receptor utilization. These findings enhance our understanding of the molecular basis of reovirus binding to JAM-A and provide clues about the mechanisms of reovirus attachment.

## MATERIALS AND METHODS

**Cells, viruses, and antibodies.** Spinner-adapted murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented to contain 5% fetal bovine serum (Gibco-BRL, Gaithersburg, Md.), 2 mM L-glutamine, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 0.25 mg of amphotericin per ml (Gibco-BRL). HeLa cells were maintained in monolayer cultures in Dulbecco's minimal essential medium (Gibco-BRL) supplemented to contain 10% fetal bovine serum, L-glutamine, and antibiotics as described for L cells. Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium supplemented with fetal bovine serum, L-glutamine, and antibiotics as described for HeLa cells.

The prototype reovirus strains T1L/53, T2J/55, and T3D/55 are laboratory stocks. The field-isolate reovirus strains used in this study are shown in Table 1. Variant K, a neutralization-resistant variant of T3D/55, was selected and characterized as previously described (7, 54, 55). Viral stocks were prepared by plaque purification and passage in L cells (67). Purified virions were prepared by using second- and third-passage L-cell lysate stocks as previously described (26, 49). Viral particle concentrations were determined by measurements of the optical density at 260 nm, using a conversion factor of $2.1 \times 10^{12}$ viral particles per optical density unit (52). The particle-to-PFU ratio of stocks used for viral infectivity assays was approximately 250 to 1.

Rabbit hCAR-specific antiserum was provided by Jeffrey Bergelson (University of Pennsylvania). Rabbit polyclonal hJAM-B- and hJAM-C-specific antisera were generated as previously described (28). The murine hJAM-A-specific monoclonal antibody (MAb) J10.4 was purified from mouse ascites by using protein A-Sepharose (34). The immunoglobulin G (IgG) fractions of polyclonal rabbit antisera raised against T1L/53 and T3D/55 (71) were purified by using protein A-Sepharose (2). A mixture of these sera was capable of recognizing all strains of reovirus used in this study.

TABLE 1. Strains used for studies of JAM-A utilization by reoviruses

| Virus strain[a] | Abbreviation | GenBank accession no. | Reference |
|---|---|---|---|
| T1/Human/Ohio/Lang/1953 | T1L/53 | M35963 | 45, 50 |
| T1/Bovine/Maryland/clone23/1959 | T1C23/59 | AY862134 | 31 |
| T1/Bovine/Maryland/clone50/1960 | T1C50/60 | AY862133 | 31 |
| T1/Human/Netherlands/1/1984 | T1Neth/84 | AY862136 | 29 |
| T1/Human/Netherlands/1/1985 | T1Neth/85 | AY862135 | 29 |
| T2/Human/Ohio/Jones/1955 | T2J/55 | M35964 | 46, 50 |
| T2/Human/Netherlands/1/1973 | T2Neth/73 | AY862137 | 29 |
| T2/Human/Netherlands/1/1984 | T2Neth/84 | AY862138 | 29 |
| T3/Human/Ohio/Dearing/1955 | T3D/55 | NC_004277 | 46, 50 |
| T3/Human/Wash.D.C./clone93/1955 | T3C93/55 | L37675 | 31 |
| T3/Human/Wash.D.C./clone87/1957[b] | T3C87/57 | L37677 | 48 |
| T3/Bovine/Maryland/clone18/1961 | T3C18/61 | L37684 | 31 |
| T3/Murine/France/clone9/1961 | T3C9/61 | L37676 | 31 |

[a] Strain nomenclature is as follows: serotype/species of origin/place of origin/strain designation/year of isolation.
[b] This strain has also been designated T3/Human/Wash.D.C./Abney/1957.

**Fluorescent-focus assays of viral infectivity.** Monolayers of HeLa cells in 96-well plates (Costar, Cambridge, Mass.) ($3 \times 10^4$ cells per well) were pretreated for 1 h with phosphate-buffered saline (PBS), hCAR-specific antiserum, or the hJAM-A-specific MAb J10.4 at various concentrations prior to the adsorption of virus at room temperature for 1 h. Following removal of the inoculum, the cells were washed with PBS and incubated at 37°C for 20 h to permit the completion of a single round of viral replication. Monolayers were fixed with 1 ml of methanol at −20°C for a minimum of 30 min, washed twice with PBS, blocked with 2.5% immunoglobulin-free bovine serum albumin (Sigma-Aldrich, St. Louis, Mo.) in PBS, and incubated at room temperature for 1 h with protein-A–affinity-purified polyclonal rabbit antireovirus serum at a 1:800 dilution in PBS–0.5% Triton X-100. The monolayers were washed twice with PBS–0.5% Triton X-100 and incubated with a 1:1,000 dilution of goat anti-rabbit immunoglobulin conjugated with the Alexa Fluor 546 fluorophore (Molecular Probes, Eugene, Oreg.). The monolayers were washed twice with PBS, and infected cells were visualized by indirect immunofluorescence using an Axiovert 200 inverted microscope modified for fluorescence microscopy (Carl Zeiss, New York, N.Y.). Infected cells were identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus. No background staining of uninfected control monolayers was noted. Reovirus antigen-positive cells were quantified by counting the fluorescent cells in at least three random fields of view per well in triplicate at a magnification of ×20.

**Transient transfection and infection of CHO cells.** CHO cells were transiently transfected with an empty vector or with plasmids encoding receptor constructs by the use of Lipofectamine PLUS reagent (Invitrogen, San Diego, Calif.) as previously described (3). After 24 h of incubation to allow receptor expression, transfected cells were allowed to adsorb to the virus at a multiplicity of infection (MOI) of 1 fluorescent focus unit (FFU) per cell, incubated for an additional 20 h, fixed with methanol, and stained for reovirus proteins by use of an anti-reovirus serum at a 1:800 dilution. Images were captured at a magnification of ×20 with a Zeiss Axiovert 200 inverted microscope.

**Flow cytometric analysis of receptor expression.** CHO cells were transiently transfected with receptor constructs, incubated for 24 h, and detached from the plates by incubation with 20 mM EDTA in PBS. Cells ($10^6$) were incubated with the hCAR-specific antiserum, the hJAM-A-specific MAb J10.4, or an antibody specific for hJAM-B or hJAM-C (28), washed with PBS, and incubated with a phycoerythrin-conjugated goat anti-rabbit or anti-rat IgG secondary antibody (Molecular Probes) at a 1:1,000 dilution on ice for 30 min. The cells were washed twice with PBS and analyzed with a FACScan flow cytometer (Becton-Dickinson, Palo Alto, Calif.).

**Sequence analysis of the S1 gene.** Viral genomes were extracted from infected L-cell lysates by the use of Trizol (Life Technologies, Rockville, Md.) according to the protocol supplied by the manufacturer. S1 gene segments were amplified by reverse transcription-PCR (RT-PCR) using 10 U of avian myeloblastosis virus reverse transcriptase (Promega Biosciences, San Luis Obispo, Calif.), 2.5 U *Taq* DNA polymerase (Promega Biosciences), and primers complementary to the 5′ and 3′ nontranslated regions of the S1 genes of the reovirus prototype strains. The type 1 S1 forward primer was 5′ GGATCCGCTATTCGCGCCTATGG

ATG, and the reverse primer was 5′ GGGTTCGCGCTAGATTCA. The type 2 S1 forward primer was 5′ GCTATTCGCACTCATGTCGGATCTAGTGC AGC, and the reverse primer was 5′ GATGAGTCGCCACTGTGCCGAGT GGA. The type 1 5′ forward primer contained nucleotides that resulted in a primer-derived sequence for the first two amino acids (M and D), and the type 2 5′ forward primer contained nucleotides that resulted in a primer-derived sequence for the first six amino acids (M, S, D, L, V, and Q). The amplification products were cloned into the pCR 2.1 vector (Invitrogen). Sequences of at least two independent RT-PCR clones for each S1 gene segment were determined by automated sequencing.

**Phylogenetic analysis of S1 gene nucleotide sequences.** Sequences were aligned by using the program ClustalX (62). Phylogenetic trees were constructed from variations in the σ1-encoding S1 gene nucleotide sequences by the maximum parsimony method using the heuristic search algorithm within the program PAUP v4.0b10 (58). Trees were rooted at the midpoint. The branching orders of the phylograms were verified statistically by resampling the data 1,000 times in a bootstrap analysis using the branch and bound algorithm as applied in PAUP.

**Sequence alignment and structural modeling methods.** Sequences were aligned by using the program ClustalW (http://www.ebi.ac.uk/clustalw/). Alignments were rendered in ALSCRIPT (5), using different colors to highlight different degrees of sequence similarity. Sequence changes were mapped onto the crystal structure of T3D/55 σ1 (16) by using the program GRASP (42).

## RESULTS

**JAM-A serves as a receptor for prototype strains of the three reovirus serotypes.** Our previous work indicated that JAM-A serves as a receptor for the prototype reovirus strains T1L/53 and T3D/55 (3). To confirm these observations and to test whether JAM-A is used as a receptor by T2J/55, we treated HeLa cells with PBS, an hCAR-specific antiserum as a control, or the hJAM-A-specific MAb J10.4 prior to viral adsorption. Infected cells were quantified by indirect immunofluorescence using an antireovirus serum (Fig. 1A). The treatment of cells with the CAR-specific antiserum had no effect on the capacity of the prototype strains to infect HeLa cells. In sharp contrast, the treatment with MAb J10.4 resulted in a concentration-dependent inhibition of infection for all three strains. The minimum concentrations of MAb J10.4 required to reduce the infectivities of these strains by 50% were between 0.1 and 1.0 μg per ml (Fig. 1B). The infectivities of all three strains were reduced approximately 90% following the treatment of cells with 100 μg per ml MAb J10.4.

**JAM-B and JAM-C do not serve as receptors for prototype strains of reovirus.** JAM-A is the only JAM family member tested to date that functions as a receptor for T1L/53 (44). To determine whether other JAM family members in addition to JAM-A serve as reovirus receptors for other reovirus prototype strains, we transfected CHO cells, which are poorly permissive for reovirus infection (24), with a cDNA encoding hJAM-A, hJAM-B, or hJAM-C. Cells also were transfected with hCAR as a negative control. After confirmation of the cell surface expression of the receptor constructs (Fig. 2A), the transfected cells were tested for the capacity to support reovirus infection. Infected cells were quantified by indirect immunofluorescence using an antireovirus serum (Fig. 2B). Only CHO cells transfected with hJAM-A were capable of supporting an efficient infection of each of the three prototype reovirus strains, whereas cells transfected with hJAM-B and hJAM-C did not support the infection of any of these strains in excess of that supported by cells transfected with hCAR (Fig. 2C). Therefore, the JAM family member JAM-A, but not JAM-B or JAM-C, functions as a receptor for prototype strains of reovirus.

**JAM-A serves as a receptor for field-isolate strains of reovirus.** Strains of each of the three reovirus serotypes have been isolated from many mammalian hosts over a period in excess of 50 years (29, 31). A type 3 reovirus strain isolated from the cerebrospinal fluid of a child with meningitis is capable of using JAM-A as a receptor (64). However, the receptor-binding properties of other field-isolate strains have not been reported. To determine whether JAM-A is used as a receptor by other field-isolate strains of reovirus, we transfected CHO cells with hJAM-A, hJAM-B, or hJAM-C and tested them for the capacity to support reovirus infection. Ten strains, encompassing four type 1, two type 2, and four type 3 viruses (Table 1), were used in these experiments. In parallel with the findings for prototype reovirus strains, each of the field-isolate strains tested was capable of utilizing hJAM-A, but not hJAM-B or hJAM-C, as a receptor (Fig. 3).

**Analysis of deduced σ1 amino acid sequences for JAM-A-binding reovirus strains.** To gain insight into the σ1 residues that mediate interactions between reoviruses and JAM-A, we analyzed the amino acid sequences of the σ1 proteins of the 3 prototype and 10 field-isolate strains chosen for study. For these experiments, we determined the σ1-encoding S1 gene sequences of the strains T1C23/59, T1C50/60, T1Neth/84, T1Neth/85, T2Neth/73, and T2Neth/84 and compared these sequences to those reported previously (Table 1). RT-PCR primers complementary to the nontranslated regions of the type 1 and type 2 S1 genes were designed to facilitate the amplification of entire S1 genes from infected L-cell lysates.

To define the evolutionary relationships of the S1 gene sequences determined for this study with those of the other reovirus strains sequenced to date, we constructed phylogenetic trees by using variation in the σ1-encoding S1 gene nucleotide sequences and the maximum parsimony method as applied in the program PAUP (Fig. 4). The most noteworthy feature of the S1 phylogenetic tree is that the strains clustered into distinct lineages based on their serotypes. A phylogenetic tree generated by using the same data set and the neighbor-joining algorithm of the phylogenetic analysis program MacVector (MacVector 2001, version 7.1.1) had a topology identical to that of the tree generated by PAUP (data not shown). Therefore, our phylogenetic analysis indicates that the S1 genes of reovirus strains cluster tightly into three lineages defined by serotype. Concordantly, changes in the deduced amino acid sequences of the σ1 protein within a given serotype are confined to a small number of residues. Since each of the strains investigated for this study was capable of using JAM-A as a receptor, the locations of these changes provide clues about areas that can vary in surface structure without impeding the capacity to engage this molecule. Thus, these changes define areas that are unlikely to interact with JAM-A.

**Sequence variability within type 3 σ1 protein.** Structural information is available for the T3D/55 σ1 protein (16). We therefore carried out a structure-based comparison of the deduced amino acid sequences of the σ1 proteins of type 3 field-isolate strains with that of the prototype T3D/55 to define regions of conserved and variable sequences within a serotype (Fig. 5). Substantial variability is seen between residues 240 and 250, a region that lies just N-terminal to the first β-spiral repeat in the crystallized fragment and is disordered in the crystal structure. The σ1 fragment was obtained by trypsin

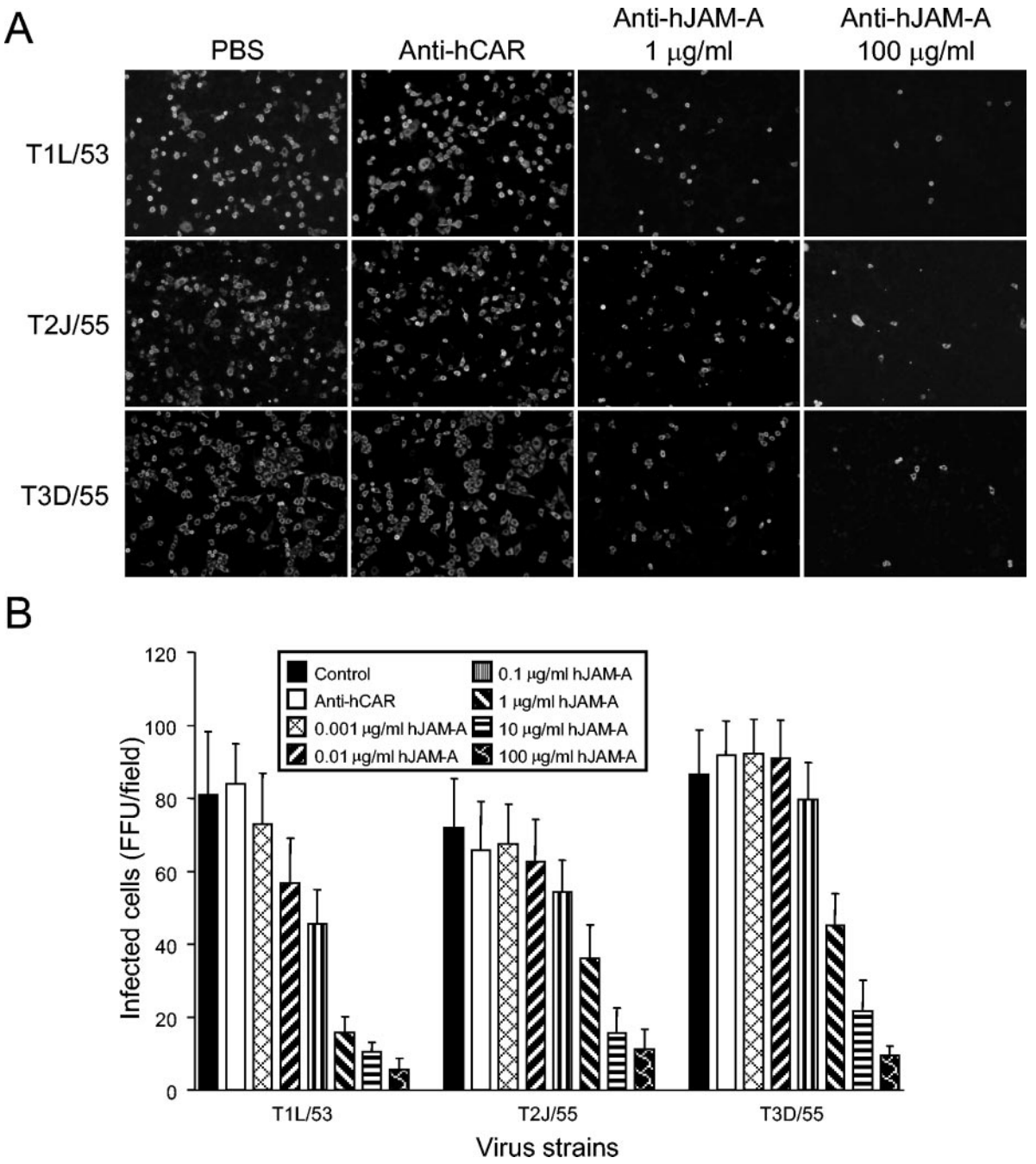



FIG. 1. JAM-A blockade reduces infection of prototype reovirus strains. HeLa cells at equivalent degrees of confluence were pretreated with PBS, an hCAR-specific antiserum as a control, or the hJAM-A-specific MAb J10.4 prior to adsorption with T1L/53, T2J/55, or T3D/55 at an MOI of 0.1 FFU per cell. After incubation for 20 h, the cells were fixed and permeabilized with methanol. Newly synthesized viral proteins were detected by the incubation of cells with a polyclonal rabbit antireovirus serum followed by incubation with an anti-rabbit immunoglobulin–Alexa-546 serum for the visualization of infected cells by indirect immunofluorescence. (A) Representative fields of view. (B) Reovirus-infected cells were quantified by counting fluorescent cells in a minimum of three random fields of view per well for three wells at a magnification of ×20. The results are presented as the mean FFU per field. Error bars indicate standard deviations.

cleavage of a longer construct after residue Arg245 (16). The fact that trypsin cleaves σ1 at only this position suggests that Arg245 lies in an exposed loop that likely possesses some flexibility. Exposed areas in the second and third β-spiral repeats of the crystallized fragment also contain several substitutions. Because these areas are variable, they are unlikely to contribute significantly to JAM-A binding.

Most of the remaining substitutions are located at the top of the σ1 trimer, forming a highly variable "plateau" that is also unlikely to bind to JAM-A. Some of the observed variations on

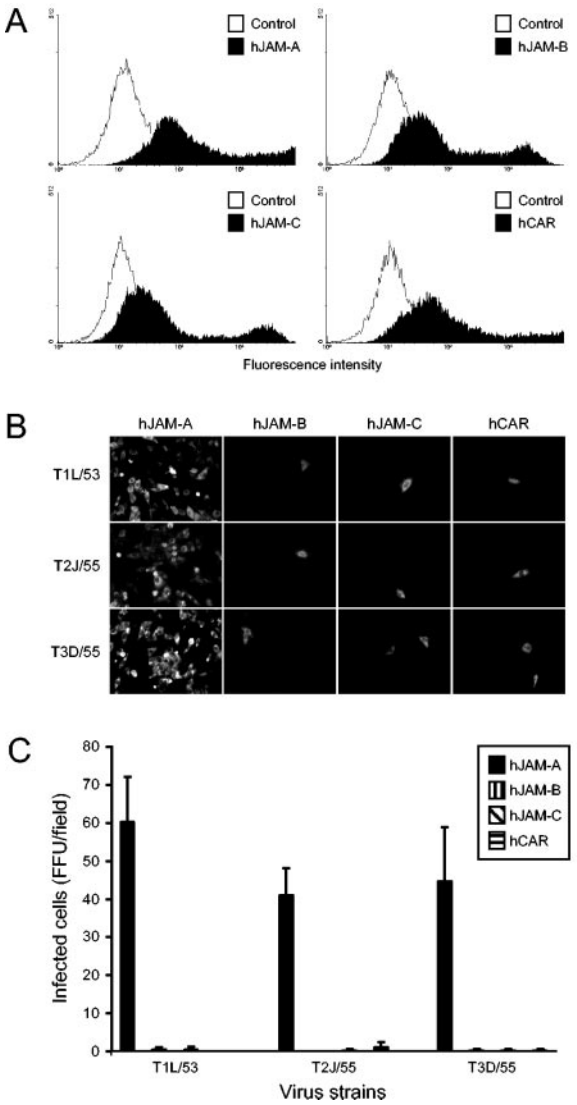



FIG. 2. CHO cells transfected with hJAM-A support growth of prototype reovirus strains. (A) CHO cells were transiently transfected with a plasmid encoding hCAR, hJAM-A, hJAM-B, or hJAM-C. Following incubation for 24 h to permit receptor expression, cells were incubated with receptor-specific MAbs, and the cell surface expression of receptor constructs was assessed by flow cytometry. (B) Transfected CHO cells at equivalent degrees of confluence were adsorbed with T1L/53, T2J/55, or T3D/55 at an MOI of 0.1 FFU per cell. Reovirus proteins were detected by indirect immunofluorescence at 20 h postinfection. Representative fields of view are shown. Magnification, ×20. (C) Reovirus-infected cells were quantified by counting fluorescent cells in five random fields of view per well for three wells. The results are presented as the mean FFU per field. Error bars indicate standard deviations.

the plateau are anticipated to alter the structure of the molecule. For example, the replacement of Ser435 with Met in T3D9/61 and T3D18/61 is likely to cause significant structural changes, as Ser435 is partially buried in the T3D/55 structure (16). Because they are exposed at the protein surface, the polymorphisms seen on the plateau may allow viral escape from antibody recognition. In contrast, the lower portion of the head domain is highly invariant, suggesting that the base of the σ1 head is primarily responsible for interactions with JAM-A.

**Sequence variability within σ1 proteins of the three reovirus serotypes.** An alignment of the deduced σ1 amino acid sequences for all of the strains chosen for study showed that only 36 of the 210 residues in the crystallized fragment of T3D/55 σ1 (16) are conserved (Fig. 6). There is substantially more variability among the serotypes than that within each serotype. Mapping of the conserved residues onto the crystal structure of T3D/55 σ1 showed that many of these residues are buried, especially those located at the base of the σ1 head-trimer interface (Fig. 6). A large fraction of the remaining conserved residues cluster in a single, solvent-exposed region at the lower edge of the β-barrel. Again, the regions that are most variable within type 3 σ1 (the β-spiral region and the "top" of the trimer) are also most variable among the different serotypes. In contrast, an extended, contiguous area of conserved residues is located at the base of the head domain, and additional, smaller areas of conservation are found along the side of this domain. Because these regions are conserved in the JAM-A-binding strains investigated here, they mark potential contact points for this receptor.

The large conserved area at the base of the head domain is formed primarily by a stretch of residues (Asn369 to Glu384 in T3D/55 σ1) within a $3_{10}$ helix and a long loop between β-strands D and E (16) (Fig. 6). This region also includes Trp421 at the end of β-strand F. The conserved region is fairly hydrophobic, with the side chains of Val371, Leu379, and Trp421 accounting for a large portion of the surface area predicted to be involved in JAM-A binding. A second, smaller cluster of conserved residues (Leu331, Trp333, Ile360, and His438 in T3D/55 σ1) lies above this putative JAM-A-binding surface, near the top of the trimer (Fig. 6). While most of the side chains of these residues are buried, the structural features of this cluster may contribute to receptor engagement. The remaining surface area of the σ1 trimer, especially near the top of the head and the head-to-head contacts, is almost entirely devoid of conserved residues.

**A neutralization-resistant variant of reovirus T3D/55 uses JAM-A as a receptor.** Variants of T3D/55 selected for their resistance to neutralization by the use of MAb 9BG5 (55) have a mutation at Asp340 or Glu419 in the σ1 head (7) (Fig. 5B). These variants have alterations in central nervous system (CNS) tropism following infections of newborn mice (54). The single mutation in variant K of σ1, Glu419 to Lys (7), segregates genetically with the altered growth and tropism of this virus in the murine CNS (32). To determine whether a neutralization-resistant variant of T3D/55 retained the capacity to use JAM-A as a receptor, we treated HeLa cells with PBS, an hCAR-specific antiserum as a negative control, or the hJAM-A-specific MAb J10.4 prior to infection with variant K. Infected cells were quantified by indirect immunofluorescence using an antireovirus serum (Fig. 7). The treatment of cells with increasing concentrations of the JAM-A-specific MAb J10.4 resulted in a dose-dependent inhibition of viral infection, while the CAR-specific antiserum had no effect on viral infectivity. At the maximal concentration of MAb J10.4 used (100 μg per ml), infectivity was nearly abolished, demonstrating that variant K is capable of using JAM-A as a receptor (Fig. 7).

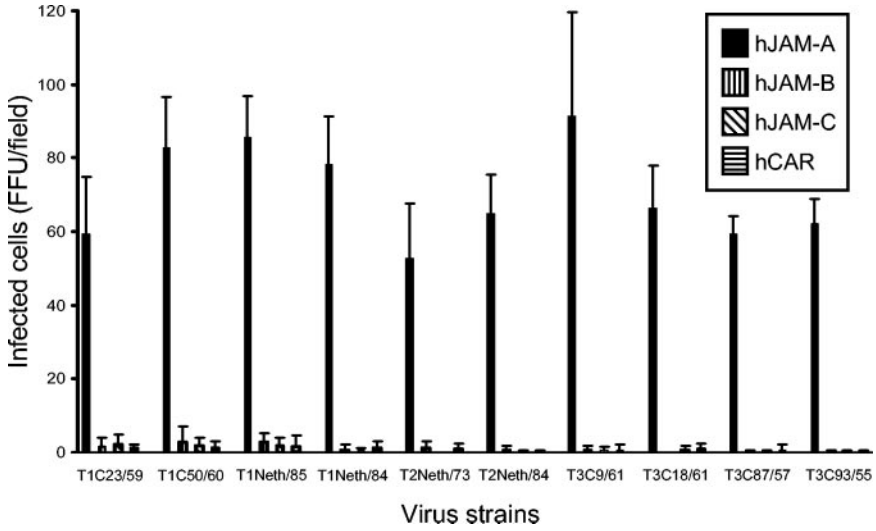



FIG. 3. Expression of JAM-A confers infectivity on field-isolate reovirus strains. CHO cells were transiently transfected with a plasmid encoding hCAR, hJAM-A, hJAM-B, or hJAM-C. Following incubation for 24 h to permit receptor expression, the cells were adsorbed with the indicated field-isolate strains at an MOI of 1 FFU per cell. Reovirus proteins were detected by indirect immunofluorescence at 20 h postinfection and quantified by counting of the fluorescent cells in three random fields of view per well for three wells. The results are presented as the mean FFU per field. Error bars indicate standard deviations.

Thus, the mechanism of the altered pathogenicity of variant K appears to be independent of JAM-A utilization.

## DISCUSSION

An examination of receptor usage by diverse virus families, including arenaviruses (11, 53), adenoviruses (9, 27, 51, 72), and measles virus (20, 35, 61), has led to the discovery that receptor usage by some viruses varies based on the viral clade, serotype, or adaptation to passaging in cell culture. We undertook this study to determine whether JAM-A is used as a receptor by both prototype and field-isolate strains of reoviruses. The results demonstrate that each of the prototype and field-isolate reovirus strains tested, regardless of their serotype, species, or geographical region of isolation, is capable of utilizing JAM-A as a receptor.

Prior to this work, sequence information for the S1 gene segments of type 1 and type 2 reovirus strains was limited to the prototype strains T1L/53 and T2J/55. In this study, we determined the S1 sequences of four type 1 and two type 2 field-isolate strains. A phylogenetic analysis of the deduced σ1-encoding S1 gene sequences revealed that these reovirus field-isolate strains are associated with discrete lineages defined by serotype. Given that all reovirus strains tested to date

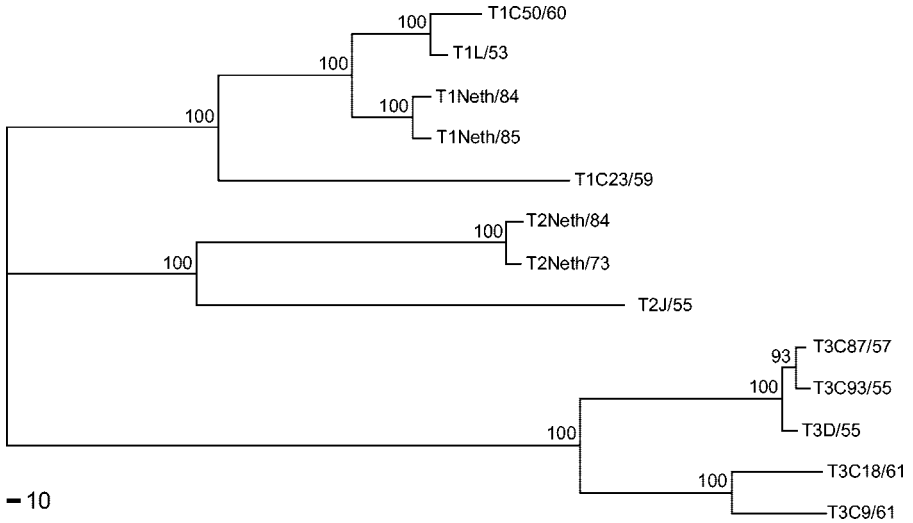



FIG. 4. Phylogenetic relationships among S1 gene nucleotide sequences of 13 reovirus strains. A phylogenetic tree for the σ1-encoding S1 gene sequences of the strains shown in Table 1 was constructed by using the maximum parsimony method as applied in the program PAUP. The tree is rooted at its midpoint. Bootstrap values of >50% (indicated as a percentage of 1,000 repetitions) for major branches are shown at the nodes. Bar, distance resulting from 10 nucleotide changes.

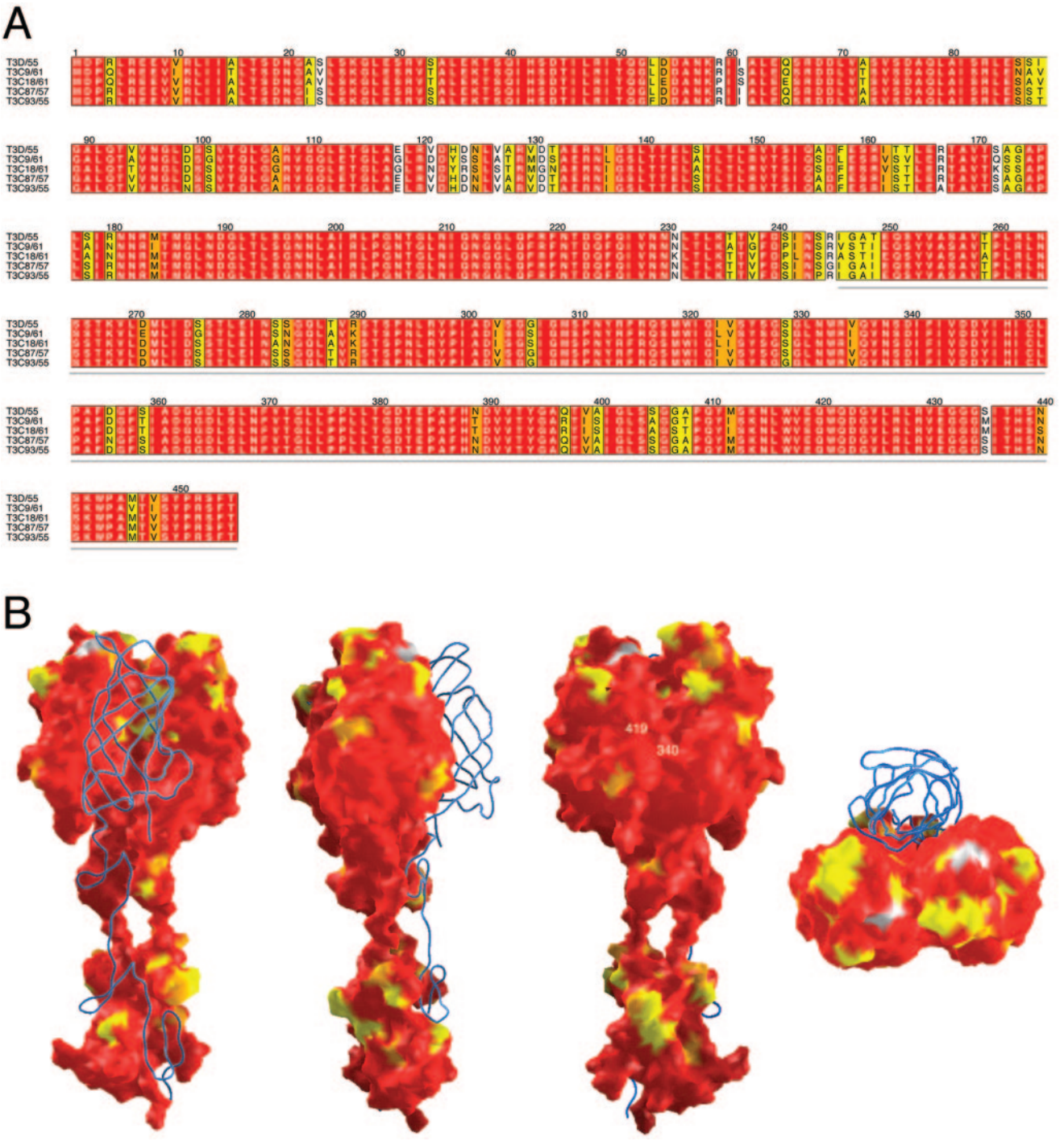
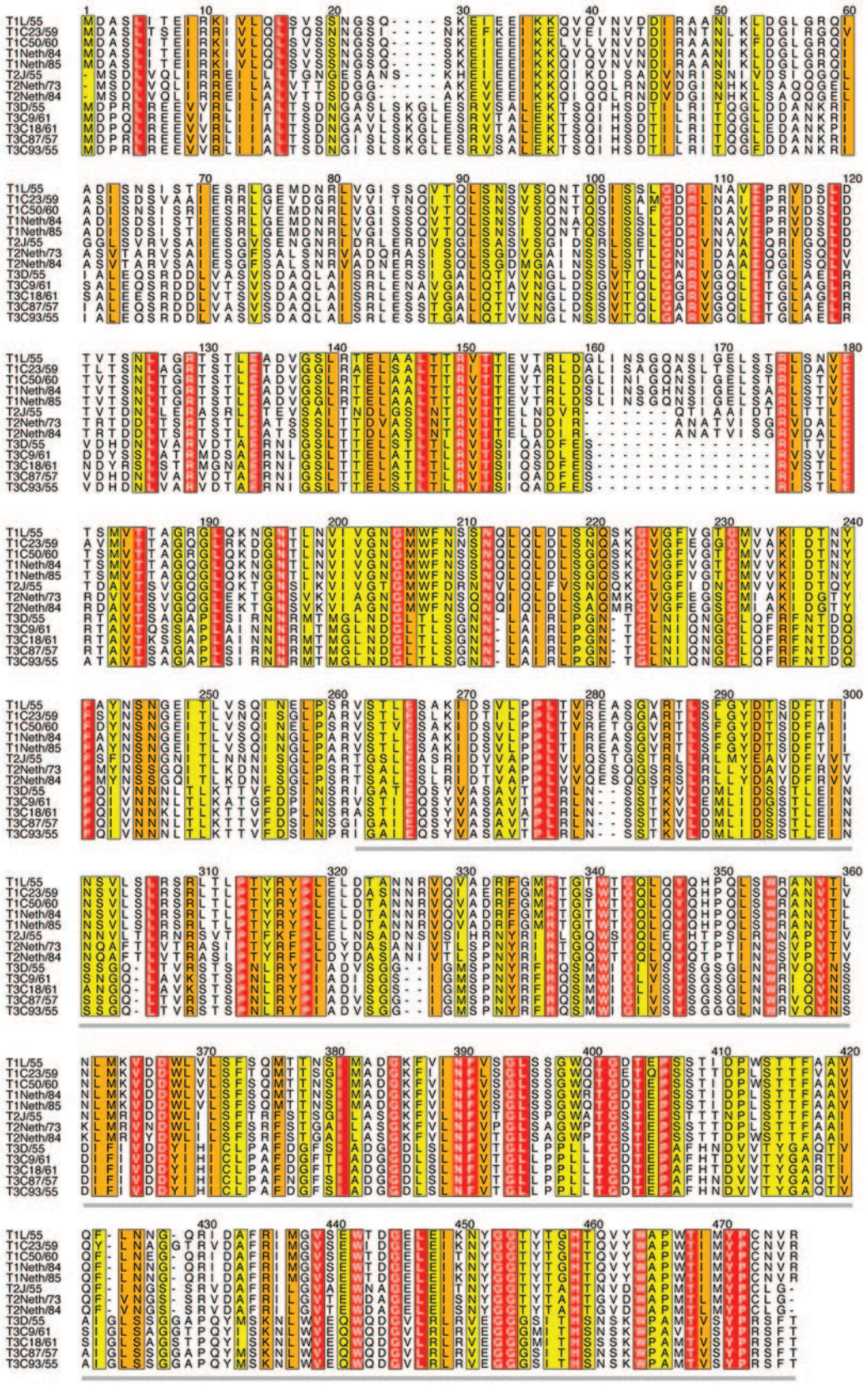



FIG. 5. Sequence conservation and structural variability within the type 3 σ1 protein. (A) Alignment of deduced amino acid sequences of the σ1 proteins of prototype strain T3D/55 and four type 3 field-isolate strains. The alignment was generated by using the program ALSCRIPT (5), with default conservation parameters applied according to the following color scheme: red, identical residues; orange, conserved residues at 80% conservation; yellow, conserved residues at 60% conservation; white, nonconserved residues. The 80% conservation threshold identifies closely related amino acids (e.g., Ile and Leu), whereas the 60% threshold identifies more distantly related amino acids (e.g., Ser and Ala, both of which have small side chains). The amino acid positions in the alignment are numbered above the sequences. The gray line indicates residues present in the crystallized fragment of T3D/55 σ1 (16). (B) Structure of the σ1 trimer, with residues colored according to the same color code as that used for panel A. Four different views are shown. For each of the views, two σ1 monomers are shown in surface representation, and the other is depicted as a blue ribbon tracing corresponding to the α-carbon backbone. The first three views each differ by 90° along a vertical axis; the fourth view shows the molecule in the third view after rotation by 90° along a horizontal axis. The positions of residues 340 and 419 are marked in the third panel from the left.

A


T1L/55
T1C23/59
T1C50/60
T1Neth/84
T1Neth/85
T2J/55
T2Neth/73
T2Neth/84
T3D/55
T3C9/61
T3C18/61
T3C87/57
T3C93/55

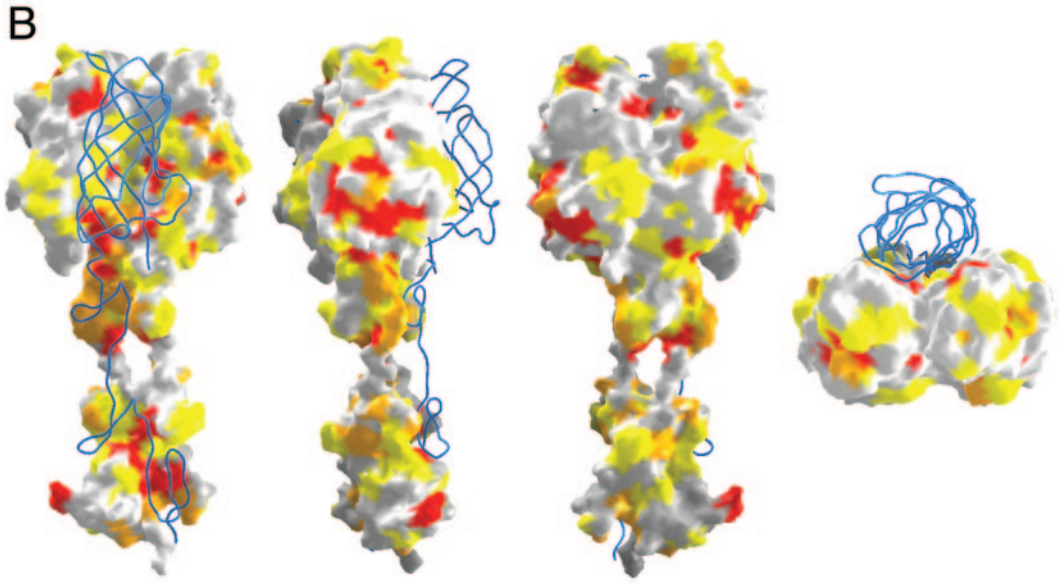



FIG. 6. Sequence conservation and structural variability within the σ1 proteins of the three reovirus serotypes. (A) Alignment of deduced amino acid sequences of the σ1 proteins of 3 prototype and 10 field-isolate reovirus strains. The alignment was generated by using ALSCRIPT and the scheme described in the legend to Fig. 5. Gaps in the aligned sequences are indicated by dots. (B) Mapping of residues onto the σ1 structure, using the same color code as that depicted in Fig. 5. The four views correspond to those in Fig. 5B.

are capable of using JAM-A as a receptor, it seems plausible that σ1 interacts with JAM-A through residues that are conserved among the serotypes, including the newly characterized type 1 and type 2 field-isolate strains. The observation that JAM-A is used as a receptor by all reovirus strains tested was unexpected since the σ1 protein is highly divergent among the three serotypes. For example, a sequence analysis of the prototype strains revealed that the σ1 head domains of T1L/53 and T2J/55 share 50% identical residues, while those of T1L/53 and T3D/55 share only 27% of their residues.

Substantial evidence has accumulated to suggest that the σ1 head domain binds to cellular receptors. Truncated forms of σ1 containing only the head domain are capable of specific cell interactions (22, 23). Concordantly, proteolysis of T3D/55 virions leads to the release of a C-terminal receptor-binding fragment of σ1 (residues 246 to 455) (13) and a resultant loss in infectivity (39). This fragment of σ1 is capable of binding to JAM-A on a biosensor surface with an affinity in the nanomolar range (3). Preliminary findings from our laboratory indicate that an even smaller fragment of T3D/55 σ1, corresponding to the head domain and a single β-spiral repeat, is capable of binding to JAM-A (K. M. Guglielmi, P. Schelling, T. Stehle, and T. S. Dermody, unpublished observations). Thus, the σ1 head promotes interactions with JAM-A that are distinct from the interactions with sialic acid mediated by the σ1 tail.

While most of the residues conserved among the σ1 proteins of the strains tested are scattered throughout the molecule, an examination of the σ1 surface revealed a single extended patch of conserved residues at the lower edge of the σ1 head. We think that this region may form part of a JAM-A-binding surface. This conserved region is formed mostly by residues in the vicinity of a long loop connecting β-strands D and E of the eight-stranded β-barrel that forms the σ1 head. Although it is easily accessible to ligands, this site is somewhat recessed into

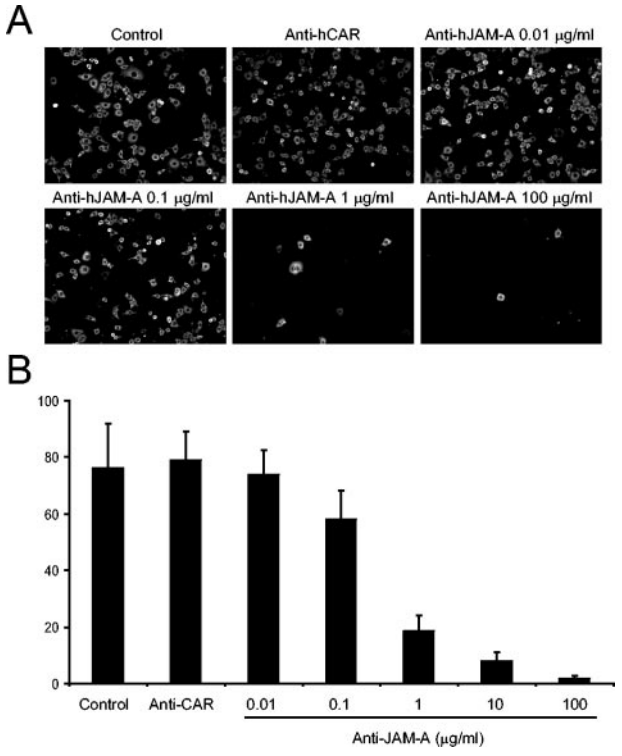



FIG. 7. JAM-A is used as a receptor for a neutralization-resistant variant of reovirus T3D/55. HeLa cells at equivalent degrees of confluence were pretreated with PBS, an hCAR-specific antiserum, or the hJAM-A-specific MAb J10.4 prior to adsorption with variant K at an MOI of 1 FFU per cell. Reovirus proteins were detected by indirect immunofluorescence at 20 h postinfection. (A) Representative fields of view. (B) Reovirus-infected cells were quantified by counting fluorescent cells in three random fields of view per well for three wells. The results are presented as the mean FFU per field. Error bars indicate standard deviations.

the protein surface and is surrounded by protruding, nonconserved residues on all three edges of the trimer. Only residues from a single monomer contribute to the putative JAM-A-binding region and its borders, and the regions are not involved in σ1 intersubunit contacts. Thus, the location of conserved residues within the trimer suggests that each σ1 monomer can independently bind to a JAM-A molecule.

Although it may serve as the primary contact point for the receptor, the putative JAM-A-binding site in σ1 is relatively small, measuring about 15 Å long and 10 Å wide. Most other interactions between viral ligands and proteinaceous receptors cover somewhat larger areas. It is therefore likely that additional regions of σ1 contribute to interactions with JAM-A. We noted that the putative JAM-A-binding site lies at the lower edge of a large, concave surface formed by β-strands B, A, D, and G of σ1. Residues on this surface, which almost entirely covers one side of the β-barrel, would easily be accessible to a receptor and do not participate in intersubunit contacts. The top of the σ1 head is formed by three prominent protrusions, with one coming from each β-barrel. These protrusions are entirely devoid of conserved residues among the serotypes and also exhibit significant sequence drift within type 3 σ1 proteins. It is therefore highly unlikely that the top of the σ1 head participates in receptor binding, again implicating regions on the side of each β-barrel as the most likely areas of contact with JAM-A.

Neutralization-resistant variants of the reovirus T3D/55 selected by using the σ1-specific MAb 9BG5 contain mutations in the σ1 head that segregate genetically with alterations in neural tropism (7, 32, 54, 55). Since reovirus tropism in the murine CNS is determined at least in part by σ1-receptor interactions (19, 60), it is possible that the antibody-selected mutations in the σ1 head alter receptor binding. However, we found that variant K, which has a Glu-to-Lys mutation at amino acid 419, uses JAM-A as a receptor. This observation suggests that the mutation in σ1 of variant K alters the interactions of this strain with cell surface receptors other than JAM-A or influences a postattachment step in reovirus replication. It is noteworthy that amino acid 419 is adjacent to the σ1 head trimer interface in the vicinity of amino acid 340 (16) (Fig. 5), which is also targeted for mutation in neutralization-resistant variants of T3D/55 (7). It is possible that mutations at these sites alter σ1 subunit interactions required for viral assembly or disassembly.

Reovirus serotypes exhibit striking differences in tropism and pathogenesis in the murine CNS. Type 1 reoviruses spread to the CNS hematogenously and infect ependymal cells (65, 70), resulting in subacute hydrocephalus (69). In contrast, type 3 reoviruses spread to the CNS by neural routes and infect neurons (38, 65, 70), causing lethal encephalitis (59, 69). An analysis of reassortant viruses containing gene segments derived from T1L/53 and T3D/55 demonstrated that the pathway of viral spread in the host (65) and tropism for neural tissues (19, 70) segregate with the σ1-encoding S1 gene. These findings suggest that σ1 determines the CNS cell types that serve as targets for reovirus infection, presumably by its capacity to bind to receptors expressed by specific CNS cells. Since all strains of reovirus tested are capable of utilizing JAM-A as a receptor, the engagement of JAM-A alone does not explain the differences in tropism and virulence displayed by the different reovirus serotypes in the murine CNS. It is possible that JAM-A serves as a serotype-independent reovirus receptor at some sites within the host and that other receptors, perhaps carbohydrate in nature, confer serotype-dependent tropism. In support of a role for cell surface carbohydrates in reovirus disease, the capacity to bind sialic acid enhances the spread of type 3 reoviruses within the host and targets the virus to bile duct epithelial cells, leading to obstructive jaundice (4). It is also possible that serotype-dependent differences in pathogenesis are influenced by one or more postbinding events.

The role of JAM-A utilization in reovirus infections in vivo is not known. JAM-A is expressed on many cell types, including intestinal epithelium, bile duct epithelium, lung epithelium, leukocytes, and CNS endothelial cells (36), which serve as sites of reovirus infection in mice (68). It will be interesting to determine whether JAM-A functions as a reovirus receptor at these sites in infected animals. Mice with a targeted disruption of the JAM-A gene are viable and fertile (12). These mice exhibit an accelerated migration of dendritic cells to lymph nodes, which is associated with enhanced contact hypersensitivity (12). No other developmental or immune abnormalities have been noted. Studies of reovirus infections using JAM-A-deficient animals should clarify the function of JAM-A in reovirus pathogenesis and disease.

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